Proposed Title for Ph.D. Research Work

Isolation, identification and functional characterization of bacteria from *Argulus bengalensis* Ramakrishna, 1951.

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1. Introduction

Symbiosis, or “living together,” generally means that there is a close association between different organisms. Of the various symbiotic associations, the most cohesive forms are found in “endosymbiosis,” in which one partner called the symbiont lives inside the body of another partner called the host. Organisms feeding exclusively on restricted diets, such as plant sap, vertebrate blood, or woody material, often possess symbiotic microorganisms. In many of these cases, specific microbes are localized in the lumen of the gut, in the hemocoel, or inside specialized cells called mycetocytes or bacteriocytes (Buchner, 1965). These microorganisms are always found in the host insects, are vertically transmitted from generation to generation (Pais et al., 2008; Hosokawa et al., 2010), and usually cannot be cultured outside the bodies of the hosts. Microorganisms play important and often essential roles in the growth and development of many insect species. In insects, these contributions include synthesis of essential nutrients, production of vitamins and sterols, processing of foods such as cellulose, food detoxification, and determination of food utilization (Douglas, 2009). Despite the importance of these microbial associations with insects there are relatively few studies that elucidate their components and their roles in the interactions. Insects that rely on nutritionally poor diets tend to possess bacterial endosymbionts. The host insects often suffer retarded growth, sterility, and/or death when they are experimentally deprived of their symbionts (Baumann et al., 1997; Buchner, 1965; Douglas, 1989).

Other than insect, the branchiurans under crustacea are exclusively parasitic. The branchiuran parasite *Argulus* is a hematophagus organism feeding on the blood of host fishes (Swanepoel and Avenant-Oldewage, 1992; Gresty et al., 1993; Saha et al., 2011). Blood-feeding insects are a good example of insects living on a nutrient-limited diet in which endosymbionts supplement essential vitamins for the host's survival (Pais et al., 2008; Hosokawa et al., 2010).Since blood is a fluid tissue rich in nutritious proteins and lipids that can be taken without enormous effort, hematophagy has evolved as a preferred form of feeding in many small animals such as worms and arthropods. *Argulus* feed by inserting a long spine-like structure (the stylet into the skin, which breaks down tissues through the secretion of enzymes. The repeated puncturing of the skin results results into substantial damage and irritation. An anticoagulant (Saha et al., 2011) and a vasodialator (Marshall et al., 2008) secreted from the glands associated with the feeding apparatus are used as antihemostatic substances by the parasite. Some other haematophagous animals which exclusively feed on blood, such as leeches, hookworms, and several species of vampire bats in spite of their diversity, these organisms all secrete anti-haemostatic and fibrinolytic compounds from their salivary glands to prevent blood coagulation during feeding and blood storage in the gut during digestion . A variety of anti-coagulant and anti-thrombotic molecules have been identified amongst blood-sucking insects. In corroboration with haematophagy, blood feeding organisms make symbiotic association with microorganisms in their gut. It is assumed that the symbionts either contribute to antihaemostatic mechanism of the parasite and prevent coagulation of blood in their gut or help in digestion of the blood providing extracellular enzymes. Sometimes the symbionts may provide nutritional supplements by synthesis of vitamins. As is the case in all vertebrates, and also many invertebrates harbor microbial populations in their digestive tracts (Trust and Sparrow, 1974; Lindsay and Harris,

1980; Lesel et al., 1986). These populations grow upon the food absorbed by the host animal and digestive secretions. But do these microorganisms furnish in return to their hosts some useful substances coming from their metabolism? Are these microorganisms only endocommensals , or does there exist a mutual relationship with their host? The bacterial flora of the endogenous sources in general, represents a very important and diversified enzymatic potential, and it seems logical to think that the enzymatic mass lodged in the digestive tract might interfere in a considerable way with a major part of the metabolism of the host animal.

Blood though rich in protein but it is nutritionally inadequate because it is deficient in B vitamins (Brook, 1964). Blood feeders with incomplete metamorphosis which feed on blood during all their nymphal stages do have symbiotic micro-organisms while, as a rule, their intestines are otherwise sterile. These arthropods are represented by bed-bugs, assassin-bugs, sucking- and chewing-lice, and ticks and mites. In addition, those blood-feeding Diptera which nourish the larval stages internally, the tsetse-flies and the Pupipara, also have symbionts (Brook, 1964). Because of the coincidence between possession of symbionts and the eating of a blood-diet, it was for long assumed that the symbionts are contributing either to digestion of the blood or to synthesis of vitamins. Evidence has been presented that an apparently symbiotic *Rickettsia* is found intracellularly in the midgut of *Culex pipiensfatigans*(Micks et al., 1961). Studies on various blood sucking arthropods revealed that the symbionts play an important role in the digestion of the parasite. Geigy et al. (1953) isolated and cultured the bacterial organisms from *Triatoma* and, the cultured symbionts produced minimal amounts of a number of growth factors, but only folic acid was produced in significant excess. Bewig and Schwarz (1955, 1956) studied the symbionts of *Haematopinussuis, Glossinasubmorsitans, Rhodniusprolixus,* and *Triatomainfestans*. All kinds of bugs suffered similar deficiency symptoms of slow growth,

crumpled wings, and failure of reproduction when reared free of their symbiont. Thus it was concluded that these symbionts play an important role in their survival. Present investigation is thus carried out to underscore the symbiotic role of endosymbionts which are harboured by an Indian species of branchiuran fish parasite *Argulus bengalensis.*

2. Rational for studies on symbiotic flora of *Argulus* sp.

A great variety of arthropods possesses symbionts-indeed we can predict that all arthropods might be found to harbor them if we but knew where and how to look for them. The symbionts undoubtedly are involved in some aspect of nutrition in its broadest sense, but this is complicated by their effect on morphology and reproduction as well as digestion. The relationship is so old and of such an intimate nature that the processes involved in many cases may be more akin to those carried out by cell organelles rather than to those of independent bacteria. Physiological relevance of the symbionts is thus a subject of study to decipher the relationship which may provide definite clue to control a parasite. The present work is thus aimed to identify and characterize the endosymbiotic bacteria and to establish their role in survival of the parasitic *Argulus.*

3. Objective

A] Isolation and characterization and identification of the endosymbionts from *Argulus sp*.

B] Stage specific CFU count through life stages of the parasite, an attempt can also be made to find out the source wherefrom the parasite is harboring the bacteria. Whether the juveniles also harbor the bacteria or later those enter into the digestive system of the adult parasite from the

environment. Besides the pathway through which these bacteria are gaining access for its better propagation through the generations has also to be found out.

C] Determination of antibiotic sensitivity of the bacterial colony.

D] Establishment of the symbiotic relationship

E] Establishment of the role of the symbionts in digestive physiology and antihaemostatic mechanism of the parasite.

F] Assay of the exoenzymes produced by the bacteria

G] Assay of vitamins synthesized by the symbionts: Since blood is a vitamin deficient diet, it is hypothesized that the symbionts will provide dietary supplementation of vitamins or other nutrients which may be essential for growth and survival of the parasite.

4. Material and methods:

4.1. Surface sterilization of parasites

50-60 numbers of adult *Argulus* sp. will be selected for the study. The parasites will be starved for 24 h in order to clear their alimentary tracts and then sterilized the body surface using 0.01% mercuric chloride before being homogenized.

4.2. Microbial culture

The homogenate of the parasites will be used after five serial 1:10 dilutions (Beveridge et al., 1991). Samples (0.1 ml) will be taken from each dilution and poured aseptically within a laminar flow on sterilized tryptone soya agar (TSA) plates, in duplicate. These culture plates will be incubated at 37 °C for 24 h. Those will be examined for the development of bacterial colonies. The well-separated colonies with apparently different morphological appearance are streaked separately on TSA plates to obtain pure cultures. Single, isolated colonies from the

streaked plates will be transferred to TSA slants. To isolate and enumerate amylase, cellulase, lipase and protease producing bacteria, diluted gut homogenate will be poured on starch-agar, carboxymethylcellulose (CMC)-agar, tributyrin-agar and peptone-gelatin-agar media containing plates, respectively. These culture plates will be incubated at 37 °C for 24 h. It is assumed that the microflora, which forms colonies on the starch plate, must have amylolytic activity. Similarly, it is assumed that the microflora grown on CMC plate, tributyrin plate and peptone-gelatin plate have cellulolytic, lipolytic and proteolytic activities respectively. By multiplying the number of colonies formed on each plate by the reciprocal of dilution, colony numbers per unit sample volume of gut homogenate will be determined.

4.3. Media composition

Tryptone Soya Agar (TSA) medium: 40 g of TSA suspended in 1000 ml distilled water. Starch-agar medium (g l^{-1}): starch, 10; Beef extract, 3;Agar, 12; pH 7. Carboxymethylcellulose-agar (CMC) medium (g l^{-1}): Carboxymethlycellulose, 10; KH_2PO_4, 4 ;Na_2HPO_4, 4; Tryptone, 2; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.001; $FeSO_4.7H_2O$, 0.004; Agar, 15; pH 7. Tributyrin- agar medium (g l^{-1}): Tributyrin-agar, 10; Peptone, 5; Agar, 15; pH 7. Peptone-gelatin- agar medium (g l^{-1}): Peptone, 5; Gelatin, 4; Beef extract, 3; Agar, 20; pH 7.

4.4. Determination of CFU

Fifty numbers of adult *Argulus* specimens will be starved for 4 h. Parasites will be surface sterilized with 0.01% mercuric chloride. Parasites will be repeatedly washed with sterile distilled water inside a laminar flow hood. Parasites will then be homogenized in sterile distilled water. Supernatant of homogenized sample will be used as inoculums and cultured within petridishes containing solidified 25 ml of Tryptone Soya Agar (TSA) medium in each. Inoculated plates will be incubated at 37 ^{o}C for 24 h. Bacterial colonies will be counted for the

determination of CFU. CFU is studied by counting number of colonies X dilution of the supernatant X 10 / ml.

4.5. Morphological characterization

Morphology of the bacteria will be studied with the aid of scanning electron microscopy. Bacterial samples from broth culture will be centrifuged and fixed with 2.5% gluteraldehyde and post fixed with OsO_4. The samples will be dehydrated with graded alcohol and finally in acetone. Samples will then be coated with gold of $100A^o$ thickness in a IB-2 Ion coater and viewed under Hitachi S- 532 Scanning Electron Microscope.

4.6. pH tolerance

The bacteria will be subjected to grow within Tryptone Soya Agar (TSA) medium at different pH. Absorbance of the culture medium will be taken in a UV/visible spectrophotometer at 650 nm wave length. Growth curve of the bacteria will be prepared plotting pH at X- axis and absorbance at Y- axis.

4.7. Temperature tolerance

Bacterial inoculums will be given inside a laminar flow hood. Inoculated culture tubes are incubated at 5^oC, 20 ^oC, 55^oC. Absorbance of the medium will be taken after 24 h, 48 h of incubation. Growth curve of the bacteria will be prepared plotting time at X axis and absorbance at Y axis.

4.8. Antibiotic sensitivity

To study the antibiotic sensitivity, the bacterial inoculums will be given within Tryptone Soya Agar (TSA) medium and antibiotic strips will be placed at different places on the medium.

The inoculated plates will be incubated at 37^{o}C for 24 h. 5 different antibiotics strips viz. doxycycline, ampicilin, chloramphenicol, norfloxacin and netilmicin are used. Clear and bacterial colony free zones surrounding the strips will indicate the susceptibility of the bacteria to specific antibiotics

4.9. First screening of the extracellular production of folate, vitamin B_{12} and thiamine

The growth of each strain in a vitamin-free medium will be checked in the first screening of the extracellular production of folate, vitamin B_{12}, and thiamine. Each strain will be grown in de Man-Rogosa-Sharpe (MRS) broth for 24 h at 37 ^{o}C without shaking. Two-milliliters of each culture will then be centrifuged at 11,000 X g for 10 min at 15^{o}C. The obtained bacterial pellet will be washed twice with sterilized phosphate-buffered saline (PBS) and re-suspended to the original volume in sterilized PBS. Each 30 ml of the cell suspension will be inoculated into 5 ml of a folate-free vitamin-free folic acid assay medium, a vitamin B_{12}free Nissui B_{12} assay medium, and a thiamine-free medium for thiamine determination, which will be each incubated at 37 ^{o}C for 24 h without shaking. Growth will be determined by measuring the optical density at 660 nm (OD660). Strains showing more than or equal to 0.5 of OD660 will be applied to a vitamin determination or the 2nd screening. After the cultures showing bacterial growth will be centrifuged at 11000 X g for 10 min at 4 ^{o}C, each obtained supernatant will be stored at -20 ^{o}C until the vitamins will be quantified.

4.10. Determination of extracellular folate

The total folate concentration, including polyglutamylfolate, will be analyzed after treating each broth with conjugase (EC 3.4.19.9: γ-glutamyl hydrolase) according to the manual for Standard Tables of Food Composition in Japan. Briefly, a 0.1 M phosphate buffer solution (20 ml, pH 6.1) will be added to each culture supernatant (2.0 ml), before being autoclaved for

15 min at 121 ^{o}C. After the test solution will have been treated with Actinase E for 3 h at 37 ^{o}C and then with conjugase prepared from hog kidney for 20 h at 37 ^{o}C, the folate concentration will microbiologically be determined by using *Lb. rhamnosus* ATCC 7469 as the growth indicator organism. The folic acid assay medium was used as an assay medium. A standard curve will be obtained by using folate at levels of 0–0.6 ng per assay tube. Growth will be determined by measuring OD660 after 20 h of incubation.

4.11. Determination of extracellular vitamin B_{12}

The vitamin B_{12} content of each culture supernatant will be determined according to the manual for Standard Tables of Food Composition in Japan. Briefly, 5 ml of a 0.57 M sodium acetate buffer (pH 4.5) and 0.2 ml of a 0.05% potassium cyanide solution will be added to 2 ml of the culture supernatant, before heating the mixture at 100 ^{o}C for 30 min. The vitamin B_{12} concentration will microbiologically be determined by using *Lb. delbrueckii* subsp. *lactis* ATCC 7830 as the growth-indicating organism. The B_{12} 'Nissui' basal medium will be used as the assay medium, a standard curve being obtained by using vitamin B_{12} at levels of 0 to 30pg per assay tube. Growth will be determined by measuring OD660 after 20 h of incubation. The alkaline heat-resistant factor will also be checked under conditions of pH 11–12 at 12^{o}C for 30 min.

4.12. Thiamine determination

The thiamine concentration of each culture supernatant will be determined according to the manual for Standard Tables of Food Composition in Japan with some modifications. A 4 ml amount of the culture supernatant will be used for screening, while 40 ml of the culture supernatant and corresponding precipitated cells will be used for a more precise estimation. Briefly, 0.4 ml of 1 M HCl will be added to 4 ml of the culture supernatant, and the mixture will be heated at 100^{o}C for 20 min. After the solution will have been treated with Taka-Diastase B

andpurified in a column of Activated Vitachange, the thiamine concentration will be determined by the thiochrome method, using post-column-HPLC with fluorescence detection. The HPLC conditions will be as follow: column, Capcell Pac C18AQ (4:6 mm i.d. X 150 mm); eluent, a mixture of 0.01 M NaH_2PO4 /0.15 M $NaClO_4$ (pH 2.2) and MeOH (98:2); flow rate, 1.0 ml/ min; column temperature, 40 ^oC; reaction solution, 0.025% $K_3Fe[CN]_6$ in 5% NaOH; flow rate of reaction solution, 0.4 ml /min; detection, fluorescence (excitation at 375 nm and emission at 440 nm).

Localization of Bacterial Symbionts in *Argulus* by Quantitative PCR assays, Fluorescent in situ hybridization can unequivocally demonstrate the bacteriome-specific localization of the symbiont.

5. Conclusion

In terms of extent of damage argulosis is a major concern of the fish farmers in India as well in other parts of the world. Several research works had been carried out for eradication of the parasite but proved to be partially effective and witnessed recurrence of parasitaemia. Development of a suitable control measure against it is thus of urgent need. The endosymbionts may be a target for management of the parasite. The present attempt may provide a definite clue for adoption a control strategy against argulosis.

Keywords: Symbiosis, endosymbionts, antibiotic sensitivity, antihaemostatic mechanism, microbial culture

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